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Extremely low frequency electromagnetic fields affect proliferation and mitochondrial activity of human cancer cell lines

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Abstract

Purpose: To date, the effects of electromagnetic fields on cell metabolism have been overlooked. The objective of the present study was to investigate the influence of extremely low frequency electromagnetic fields (ELF-EMF) over mitochondrial metabolism and the consequent impact on cancer cell growth.

Materials and methods: The effects of ELF-EMF on cancer growth were investigated in several human cancer cell lines by crystal violet assay. The modulation of mitochondrial activity was assessed by cytofluorimetric evaluation of membrane potential and by real-time quantification of mitochondrial transcription. Moreover the expression of several mitochondrial proteins and their levels in the organelle were evaluated.

Results: The long-term exposure to ELF-EMF reduced the proliferation of several cancer cell lines and the effect was associated to an increased mitochondrial activity without evident changes in ATP levels. The results of our experiments excluded a transcriptional modulation of mitochondrial respiratory complexes, rather suggesting that ELF-EMF increased the energy demand. The altered mitochondrial metabolism led to changes in mitochondrial protein profile. In fact we found a downregulated expression of mitochondrial phospho-ERK, p53 and cytochrome c.

Conclusion: The results of the present study indicate that ELF-EMF can negatively modulate cancer cell growth increasing respiratory activity of cells and altering mitochondrial protein expression.

Introduction

Humans are exposed daily to electric and magnetic fields from both natural and man-made sources. In addition to the static magnetic field of the earth (25–65 μT, from equator to poles), people are exposed to electric and magnetic fields arising from a wide variety of sources which use electrical energy at various frequencies. The electromagnetic fields (EMF) normally encountered by the population are of low magnitude and frequency, resulting from power distribution systems in which the frequencies generated are 50 Hz (in Europe and much of the world) or 60 Hz (in the US). Background 50 Hz magnetic fields in typical homes are between 0.01 and 1 μT, with appliances generating fields of 0.1–100 μT. The flux density decreases rapidly with distance from appliances, up to a distance of 1 m where the flux density is similar to background levels.

EMF of the magnitude to which we are now regularly exposed have been implicated as a contributory factor in the incidence of childhood cancers, particularly leukemia and brain cancer. However, the epidemiological studies are unable to provide a clear correlation between exposure to extremely low frequency EMF (ELF-EMF) and the development of cancers. The evaluations of carcinogenic risk made by the International Agency for Research on Cancer (IARC) has concluded that there is *limited or inadequate evidence* in humans for the carcinogenicity of ELF-EMF in relation to childhood leukaemia and all other cancers. The overall evaluation had defined the ELF-EMF as *possibly carcinogenic to humans* (*Group 2B*).

ELF-EMF affect several biological functions, such as gene expression, regulation of cell fate, and cell differentiation. ELF-EMF can also modify the biophysical properties of cell membranes, including their permeability to Ca^{2+} ions. Several studies have investigated the effects of ELF-EMF on calcium homeostasis and cell differentiation. For example in rat chromaffin cells ELF-EMF promote neurite growth by stimulating the activity of high voltage-activated (HVA) Ca^{2+} channels (Morgado-Valle et al. 1998 Morgado-Valle C,

Verdugo-Diaz L, Garcia DE, Morales-Orozco C, Drucker-Colin R. 1998. The role of voltage-gated Ca2 + channels in neurite growth of cultured chromaffin cells induced by extremely low frequency (ELF) magnetic field stimulation. Cell Tissue Res 291:217–230). Moreover, ELF-EMF enhance the neuronal differentiation of cortical neural stem cells in vitro, and this effect is mediated by the up-regulation of Cav1-channel expression and activity (Piacentini et al. 2008 Piacentini R, Ripoli C, Mezzogori D, Azzena GB, Grassi C. 2008. Extremely low-frequency electromagnetic fields promote in vitro neurogenesis via upregulation of Ca(v)1-channel activity. J Cell Physiol 215:129–139). A study on muscle cell differentiation and function carried out using C2C12 cells reported that ELF-EMF altered intracellular Ca(2+) homeostasis (Morabito et al. 2010 Morabito C, Rovetta F, Bizzarri M, Mazzoleni G, Fanò G, Mariggiò MA. 2010. Modulation of redox status and calcium handling by extremely low frequency electromagnetic fields in C2C12 muscle cells: a real-time, single-cell approach. Free Radic Biol Med 48:579–589).

The effects of ELF-EMF on cell proliferation have been explored, although with different results depending on the characteristics of the experimental model and the applied field. Some reports have concluded that ELF-EMF can promote cell proliferation (Wei et al. 2000 Wei M, Guizzetti M, Yost M, Costa LG. 2000. Exposure to 60-Hz magnetic fields and proliferation of human astrocytoma cells in vitro. Toxicol Appl Pharmacol 162:166–176, Manni et al. 2002 Manni V, Lisi A, Pozzi D, Rieti S, Serafino A, Giuliani L, Grimaldi S. 2002. Effects of extremely low frequency (50 Hz) magnetic field on morphological and biochemical properties of human keratinocytes. Bioelectromagnetics 23:298–305., Vianale et al. 2008 Vianale G, Reale M, Amerio P, Stefanachi M, Di Luzio S, Muraro R. 2008. Extremely low frequency electromagnetic field enhances human keratinocyte cell growth and decreases proinflammatory chemokine production. Br J Dermatol 158:1189–1196., Martinez et al. 2012 Martinez MA, Ubeda A, Cid MA, Trillo MA. 2012. The proliferative response of NB69 human neuroblastoma cells to a 50 Hz magnetic field is mediated by ERK1/2 signaling. Cell Physiol Biochem 29:675–686., Trillo et al. 2012 Trillo MA, Martinez MA, Cid MA, Leal J, Ubeda A. 2012. Influence of a 50 Hz magnetic field and of all-transretinol on the proliferation of human cancer cell lines. Int J Oncol 40:1405–1413.), whereas others have demonstrated that ELF-EMF exposure inhibits cell proliferation (Gluck et al. 2001 Gluck B, Guntzschel V, Berg H. 2001. Inhibition of proliferation of human lymphoma cells U937 by a 50 Hz electromagnetic field. Cell Mol Biol 47:OL115–117.[PubMed], Van Den Heuvel et al. 2001 Van Den Heuvel R, Leppens H, Nemethova G, Verschaeve L. 2001. Haemopoietic cell proliferation in murine bone marrow cells exposed to extreme low frequency (ELF) electromagnetic fields. Toxicol In Vitro 15:351–355., Stronati et al. 2004 Stronati L, Testa A, Villani P, Marino C, Lovisolo GA, Conti D, Russo F, Fresegna AM, Cordelli E. 2004. Absence of genotoxicity in human blood cells exposed to 50 Hz magnetic fields as assessed by comet assay, chromosome aberration, micronucleus, and sister chromatid exchange analyses. Bioelectromagnetics 25:41–48., Zhou et al. 2011 Zhou J, Ming LG, Ge BF, Wang JQ, Zhu RQ, Wei Z, Ma HP, Xian CJ, Chen KM. 2011. Effects of 50 Hz sinusoidal electromagnetic fields of different intensities on proliferation, differentiation and mineralization potentials of rat osteoblasts. Bone 49:753–761., Delle Monache et al. 2013 Delle Monache S, Angelucci A, Sanità P, Iorio R, Bennato F, Mancini F, Gualtieri G, Colonna RC. 2013. Inhibition of angiogenesis mediated by extremely low-frequency magnetic fields (ELF-MFs). PLoS One 8:e79309., Huang et al. 2014a Huang CY, Chang CW, Chen CR, Chuang CY, Chiang CS, Shu WY, Fan TC, Hsu IC. 2014a. extremely low-frequency electromagnetic fields cause G1 phase arrest through the activation of the ATM-Chk2-p21 pathway. PLoS One 9:e104732., Jung et al. 2014 Jung IS, Kim HJ, Noh R, Kim SC, Kim CW. 2014. Effects of extremely low frequency magnetic fields on NGF induced neuronal differentiation of PC12 cells. Bioelectromagnetics 35:459–469.). The effects of electromagnetic fields have been tested on cancer, both in in vivo and in vitro models. Although the parameters of the applied field are different in each study, the most frequent conclusion is that ELF-EMF reduces tumor cell growth (Berg et al. 2010 Berg H, Günther B, Hilger I, Radeva M, Traitcheva N, Wollweber L. 2010. Bioelectromagnetic field effects on cancer cells and mice tumors. Electromagn Biol Med 294:132–143. Jiménez-García et al. 2010 Jiménez-García MN, Arellanes-Robledo J, Aparicio-Bautista DI, Rodríguez-Segura MA, Villa-Treviño S, Godina-Nava JJ. 2010. Anti-proliferative effect of extremely low frequency electromagnetic field on preneoplastic lesions formation in the rat liver. BMC Cancer 10:159., Sadeghipour et al. 2012 Sadeghipour R, Ahmadian S, Bolouri B, Pazhang Y, Shafiezadeh M. 2012. Effects of extremely low-frequency pulsed electromagnetic fields on morphological and biochemical properties of human breast carcinoma cells (T47D). Electromagn Biol Med 31:425–435.). Numerous cellular and molecular mechanisms

involved in these effects have been described. To cite a few, NF-KB, PKC and ERK 1/2 signaling, cell membrane morphology and kinase activity, calcium fluxes, ROS generation, and cell cycle pathways are affected by ELF-EMF. Little is known about the effects exerted by ELF-EMF on mitochondrial metabolism. Few studies have investigated the effects on apoptosis-related proteins (Nikolova et al. 2005 Nikolova T, Czyz J, Rolletschek A, Blyszczuk P, Fuchs J, Jovtchev G, Schuderer J, Kuster N, Wobus AM. 2005. Electromagnetic fields affect transcript levels of apoptosis-related genes in embryonic stem cell-derived neural progenitor cells. FASEB J 19:1686–1688.) and ROS production (Luukkonen et al. 2014 Luukkonen J, Liimatainen A, Juutilainen J, Naarala J. 2014. Induction of genomic instability, oxidative processes, and mitochondrial activity by 50Hz magnetic fields in human SH-SY5Y neuroblastoma cells. Mutat Res Fundam Mol Mech Mutagen 760:33–41.). Moreover it has been reported that sperm exposure to ELF-EMF resulted in a progressive and significant increase of mitochondrial membrane potential (Iorio et al. 2011 Iorio R, Delle Monache S, Bennato F, Di Bartolomeo C, Scrimaglio R, Cinque B, Colonna RC. 2011. Involvement of mitochondrial activity in mediating ELF-EMF stimulatory effect on human sperm motility. Bioelectromagnetics 32:15–27.). Our recent study on mitochondrial metabolism has pointed out the central role of this organelle not only in energy production but also in proliferation control. In fact we have demonstrated that increasing mitochondrial activity hampers cancer cell proliferation, because mitochondrial intermediates are channeled into the oxidative metabolism and do not support biosynthetic pathways essential to proliferation (Consiglio et al. 2014 Consiglio M, Destefanis M, Morena D, Foglizzo V, Forneris M, Pescarmona G, Silvagno F. 2014. The vitamin D receptor inhibits the respiratory chain, contributing to the metabolic switch that is essential for cancer cell proliferation. PLoS One 9:e115816.). We wondered if this metabolic switch could occur also in cancer cells exposed to electromagnetic fields.

The aim of this study was to examine the effects of ELF-EMF on cancer cell proliferation and mitochondrial activity. The frequency and intensity of the applied field was similar to the background electromagnetic fields found in homes, generated by the geomagnetic field and electrical appliances. The influence on proliferation was investigated in several human cancer cell lines, and then the modulation of mitochondrial activity, transcription and protein expression was evaluated. We found that the long-term exposure of the cancer cells to an electromagnetic field of 50 Hz and 12 μT hampers cell proliferation, assessed as change in cell number, along with a perturbation in mitochondrial activity and in mitochondrial protein levels.

Materials and methods

ELF-EMF exposure system

The experimental setup (shown in Supplementary Figure S1, available online) was composed of two independent couples of coaxial coils made of 200 loops (formed of copper wire, 0.3 mm in diameter) each loop being 2.5 cm in length (with a resulting loop density of 8000 loops per meter) and wound into a tightly packed plastic frame. The frame had a cylindrical shape with an outer radius of 8 cm, and the distance between the two coaxial coil couples was 8 cm. The cell culture dish was placed in the central part of the apparatus for magnetic irradiation. The experimental setup was placed in the incubator inside a box that shielded the apparatus from the background magnetic field; the inside residual magnetic field measured around 1–2 μT. The box was made of stainless steel and the shield was produced by a 2 mm thick inner layer of mu-metal and an outer layer produced by a special aluminium-free alloy provided by the company G-Iron of Arezzo (Italy). The inner coils (7.5 cm radius) were connected to an AC current generator. The AC current signal was a square wave beginning from zero up to a chosen positive value, with 50 steps each second and a duty cycle of 50%, the upper value was fixed in order to provide an AC magnetic field of 50 Hz and 12 μT rms in the irradiation volume. The outer coils were supplied with a DC current and they provided a constant magnetic field of 45 μT. This value was chosen because in the Turin area, and particularly in the building where the measurements were carried out, the average value of the earth's magnetic field is about 45 ± 5 μT. Therefore, the DC magnetic field inside the box reproduced, in a controlled way, the effect of the average magnetic field of the environment. In the setup irradiation volume, the point-to-point variation for the total magnetic field was up to ± 5% of the nominal value. Inside the box, the temperature and humidity were homogenous to the rest of the incubator.

Cell culture and proliferation assay

The SKBR3 human breast cancer cell line, GTL16 human gastric cancer cell line, HT29 human colon cancer cell line and the A375P human melanoma cell line were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA), and were cultured in Dulbecco's modified Eagle's medium (DMEM) that had been supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin-streptomycin) at 37 °C in a humidified atmosphere containing 5% CO_2. All culture reagents were from Sigma-Aldrich (Sigma, St Louis, MO, USA). The effect of ELF-EMF on the growth of the different human cell lines was determined by colorimetric measurement of cell numbers by crystal violet staining. Different amounts of cells (2000, 1000 or 500) were seeded on 96-multiwell plates, taking into account their different proliferation rate, and they were cultured for 7 days in standard conditions or exposed to ELF-EMF. At the end of this period, the cells were fixed for 15 min with 11% glutaraldehyde and the plates were washed three times, air-dried and stained for 20 min with a 0.1% crystal violet solution. The plates were then extensively washed and air-dried prior to solubilization of the bound dye with a 10% acetic acid solution. The absorbance was determined at 595 nm. The data collected from six wells were averaged for each experimental condition, and each experiment was repeated three times. The variation in cell number was considered proportional to cell proliferation because no signs of apoptotic cells were evident by cytofluorimetric assay of cells stained with propidium iodide (PI) (Sigma-Aldrich, USA). Cells analyzed on a FACSCalibur flow cytometer using the Cell Quest acquisition and analysis software (BD, Basle, Switzerland) never showed the sub-G0 peak indicative of DNA fragmentation.

Extract preparation and Western blotting analyses

Subcellular fractionation and Western blotting analyses were conducted, as previously described (Silvagno et al. 2013 Silvagno F, Consiglio M, Foglizzo V, Destefanis M, Pescarmona G. 2013. Mitochondrial translocation of vitamin D receptor is mediated by the permeability transition pore in human keratinocyte cell line. PLoS One 8:e54716.). The protein content of the total extracts and mitochondrial fractions was quantified using the DC protein assay (Bio-Rad, Hercules, CA, USA), and 50 μg of total lysates or 30 μg of the mitochondrial fractions were separated using 10% SDS-PAGE and analyzed using Western blotting. The proteins were immunostained with the indicated primary antibodies for 1 h at room temperature and detection of the proteins of interest was performed using peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL, USA), followed by ECL detection (ECL detection kit, Perkin Elmer Life Science, Foster City, CA USA). The mouse anti-ERK (MAB1576) and anti-pERK (MAB1825) antibodies were purchased from R&D Systems (Minneapolis, MN, USA). The mouse anti-c-ErbB2/c-Neu (Ab-3) was from MILLIPORE Temecula, CA, USA, and the mouse anti-cytochrome c (65981A) antibody was from BD Biosciences Pharmingen (San Diego, CA, USA). The anti-VDAC (anti-porin 31HL) monoclonal antibody was purchased from Calbiochem (La Jolla, CA, USA). The rabbit anti-PARP (sc-7150) and anti-p53 (sc-6243) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

RNA extraction and real-time PCR

RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and 1 μg of total RNA that had been treated with DNase (Roche, CH) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's recommended protocol. Real-time PCR was performed using iQ SYBR Green (Bio-Rad) with the following primers:

COXII: fwd 5'-CGACTACGGCGGACTAATCT, rev 5'-TCGATTGTCAACGTCAAGGA; COXIV: fwd 5'-CGAGCAATTTCCACCTCTGT, rev 5'-GGTCAGCCGATCCATATAA;

β-actin: fwd 5'-CATGTACGTTGCTATCCAGGC, rev 5'-CTCCTTAATGTCACGCACGAT.

Beta-actin was used as an internal control. The real-time PCR parameters were as follows: Cycle 1, 50 °C for 2 min; cycle 2, 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and then 60 °C for 1 min. The 2-ΔΔCT method was used to analyze the data.

Measurement of the mitochondrial membrane potential (*ΔΨm*)

JC-1, a mitochondrial dye that stains the mitochondria in living cells in a membrane potential-dependent fashion, was used to determine ΔΨm. JC-1 is a cationic dye that indicates mitochondrial polarization by shifting its fluorescence emission from green (530 nm) to red (590 nm). The cells were harvested by trypsinization, washed with PBS and incubated with JC-1 (2 μg/ml final concentration, Biotium, Hayward, CA, USA) at 37 °C for 30 min. After washing, JC-1 accumulation was determined using flow cytometric analysis. The amount of JC-1 retained by 10,000 cells per sample was measured at 530 nm (FL-1 green fluorescence) and 590 nm (FL-2 red fluorescence) using a flow cytometer and analyzed using Cell Quest Alias software. The ratio of FL2/FL1 was evaluated to determine ΔΨm. In order to exclude a variation in mitochondrial number or size, mitochondrial mass was evaluated analyzing the expression of VDAC (a mitochondrial resident porin) by Western blotting, a method previously employed to estimate mitochondrial mass (Garrabou et al. 2007 Garrabou G, Soriano A, López S, Guallar JP, Giralt M, Villarroya F, Martínez JA, Casademont J, Cardellach F, Mensa J, Miró O. 2007. Reversible inhibition of mitochondrial protein synthesis during linezolid-related hyperlactatemia. Antimicrob Agents Chemother 51:962–967., Morscher et al. 2015 Morscher RJ, Aminzadeh-Gohari S, Feichtinger RG, Mayr JA, Lang R, Neureiter D, Sperl W, Kofler B. 2015. Inhibition of neuroblastoma tumor growth by ketogenic diet and/or calorie restriction in a CD1-Nu mouse model. PLoS One 10:e0129802.). The levels of VDAC in total lysates of control and treated cells were not significantly different.

Evaluation of ATP levels

The amount of ATP in mitochondria, prepared by subcellular fractionation, was measured with the ATP Bioluminescent Assay Kit (FL-AA, Sigma), using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA). ATP was quantified as relative light units (RLU); data were converted into nmol ATP/mg mitochondrial proteins.

Measurement of intracellular lactate dehydrogenase (*LDH*) *activity*

The cells were harvested by scraping and sonicated on ice with two 10 s bursts Aliquots of cell lysate were supplemented with a reaction mixture for the measurement of LDH, as previously described (Beutler 1975 Beutler R. 1975. Red cell metabolism. A manual of biochemical methods, 2nd ed. New York: Grune & Stratton.). Enzymatic activity, measured spectrophotometrically as absorbance variation at 340 nm (37 °C), was expressed as μmol NADH oxidized/min/mg cell protein.

Statistical analyses

The data are presented as the means ± SD. Statistical analysis of the data was performed using an unpaired, two-tailed Student's *t*-test; $p < 0.05$ was considered to be significant.

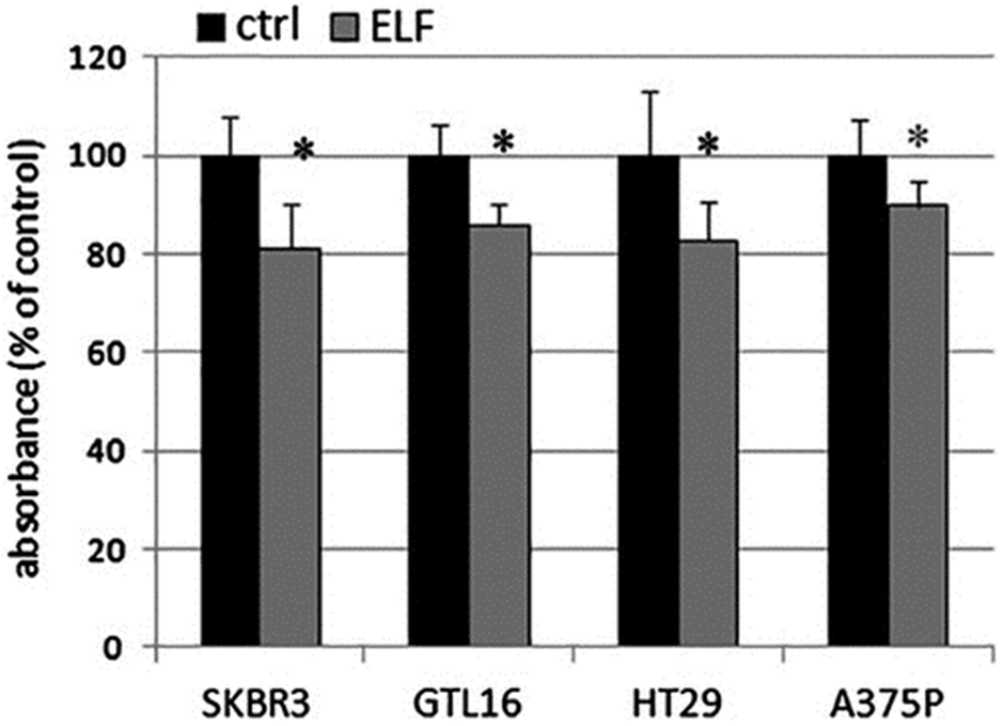
Results

Effect of ELF-EMF on proliferation

Cell proliferation was assessed in several human cancer cell lines exposed to ELF-EMF for 7 days. The analyzed cancer cells SKBR3, GTL16, HT29 and A375P were highly proliferating in regular culture conditions, and were representative of different kind of tumors: respectively, breast, gastric, colon and melanoma human cancers. We carried out the crystal violet assay on control and treated cells, and we found that all

cell lines were affected by electromagnetic field exposure. As shown in Figure 1, after 7 days of treatment cell growth was significantly inhibited. Two cell lines, the SKBR3 breast cancer cell line and HT29 colon cancer cell line, were then selected to investigate the alterations in mitochondrial metabolism and mitochondrial protein expression triggered by ELF-EMF.

Figure 1. Exposure to electromagnetic field inhibits the proliferation of several human cancer cell lines. The cells were subjected to crystal violet assay after 7 days of growth in presence or absence of ELF-EMF. The cells were stained and the absorbance values of the treated cells (ELF) are expressed as the percentage of their respective controls (ctrl). The data are expressed as the means ± SD of three independent experiments. *$p < 0.05$ compared to the control.

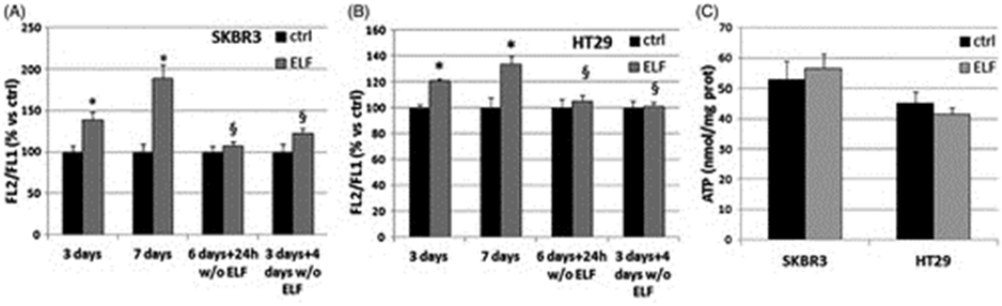


Effect of ELF-EMF on mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) is a major parameter which reflects mitochondrial functionality and it is dependent on electron transport chain activity. In order to evaluate whether the inhibitory effect induced by ELF-EMF on cell growth was associated with changes in ΔΨm, we assessed the activity of mitochondria by flow cytometric analysis of JC-1 staining. Red emission of the dye is indicative of the formation of JC-1 polymers after potential-dependent aggregation in the mitochondria, reflecting normal mitochondrial membrane potential, whereas green fluorescence indicates the presence of the monomeric cytosolic form of JC-1. Variation in membrane potential was therefore determined by the ratio of red/green fluorescence intensity. When SKBR3 cells were exposed to the electromagnetic field, this ratio was increased relative to untreated control cells. The increase was significant after 3 days and even more evident after 7 days of exposure, as shown in Figure 2A. The red/green fluorescence ratio was restored at control levels when, in a set of 7 days experiments, the cells were returned to normal incubator after 6 days of ELF-EMF exposure and assayed after 24 h without treatment. The same decrease was seen when cells were exposed for 3 days and analyzed after 4 days without ELF-EMF (Figure 2A). These data led to the conclusion that the effects of ELF-EMF on mitochondrial membrane potential were reversible. The increase of mitochondrial membrane potential triggered by ELF-EMF was evident also in HT29 cells, after 3 and 7 days of exposure, as shown in Figure 2B, and reverted by the same experimental conditions, in agreement and support of what observed in SKBR3 cells. When we evaluated the impact of ELF-EMF on ATP synthesis, we found no differences in ATP levels of control and treated cells, as shown in Figure 2C. The enhanced

mitochondrial membrane potential associated to unchanged ATP levels was suggestive of a possible increase of ATP demand and consumption when cells were exposed to electromagnetic field, because ATP supply and demand are usually matched. Only mitochondrial metabolism was stimulated, whereas the cytosolic LDH activity did not change (Supplementary Figure S2); although indirectly, the assay of this key enzyme of aerobic glycolysis gave some indication that aerobic glycolysis was not consistently perturbed by ELF-EMF in these cells.

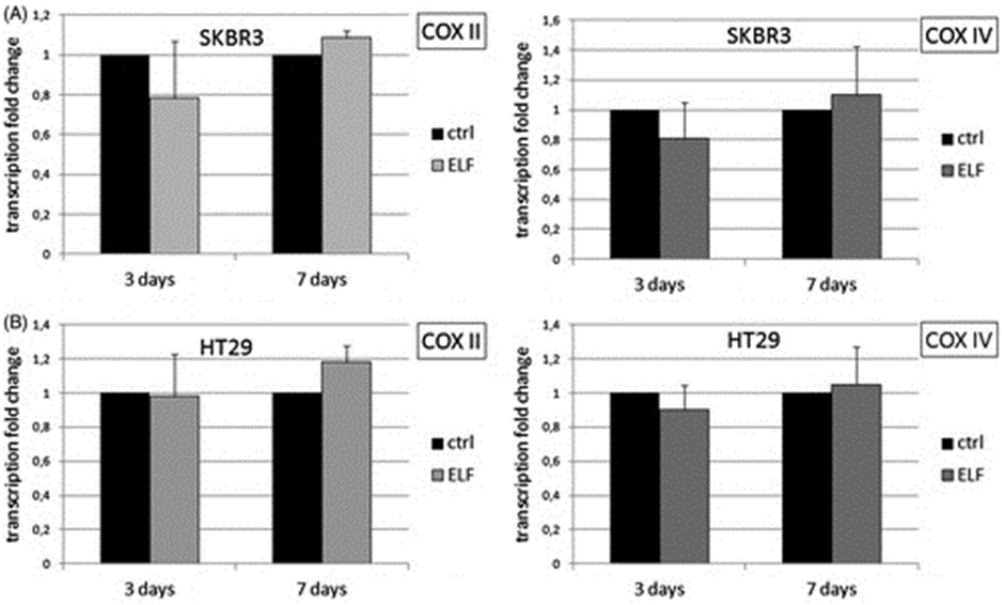
Figure 2. The electromagnetic field increases mitochondrial membrane potential. (A) SKBR3 cells were stimulated for 3 days or 7 days with ELF-EMF, or kept for 6 days in ELF-EMF and then returned to standard culture conditions for 24 h, or 3 days in ELF-EMF and 4 days without it. The mitochondrial membrane potential was examined using JC-1 cytofluorimetric evaluation. The FL-2/FL-1 ratio was calculated and the values were expressed as a percentage of the control. The results from three separate experiments are plotted on graph, where the data are expressed as the means ± SD. (B) The same experiments were repeated for HT29 cells and the results of three independent experiments are shown in graph. *$p < 0.05$ compared to the control; §$p < 0.05$ compared to ELF 7 days and 3 days. (C) ATP levels of cells untreated (ctrl) or exposed to ELF-EMF for 7 days (ELF) were measured by a chemiluminescence-based assay. Measurements were performed in triplicate and data are presented as means ± SD ($n = 3$).



Evaluation of respiratory chain mRNA transcription under ELF-EMF influence

The modulation of respiratory activity induced by ELF-EMF as measured in flow cytometry must be strictly associated to an alteration of the electron transport chain activity. The increased mitochondrial activity could be the result of an increased mRNA transcription and respiratory chain protein expression or could derive from an allosteric modulation of the respiratory complexes triggered by an increased energy demand. To identify which mechanism might be responsible for the observed increased mitochondrial activity, we measured by real time PCR the transcript abundance of two subunits of complex IV: cytochrome c oxidase subunit II (COX II) and subunit IV (COX IV). The two transcripts are of mitochondrial (the former) and nuclear (the latter) origin; with our analysis therefore we considered a possible influence of electromagnetic fields on both transcriptional events. After 3 and 7 days of exposure to ELF-EMF the treated cells did not show any significant variation in the levels of the two mRNAs when compared to control cells, as shown in Figure 3A for SKBR3 cells and in Figure 3B for HT29 cells. Thus when cells were exposed to the selected ELF-EMF, a modulation of the transcription could be excluded.

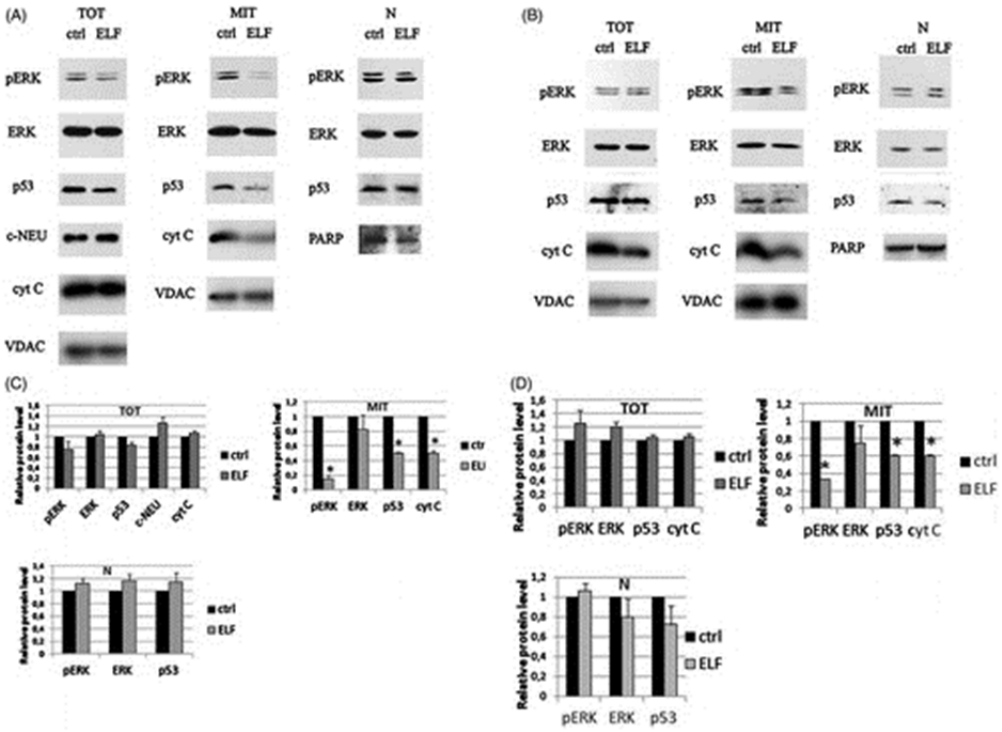
Figure 3. Mitochondrial transcription is not affected by electromagnetic field exposure. (A) Real time analysis of COX II and COX IV subunit transcript expression in control and treated SKBR3 cells. The assay was carried out after 3 days and 7 days of exposure to ELF-EMF. (B) The same analysis was performed on HT29 cells. Fold changes are plotted on the graphs as the means ± SD of three independent experiments.



Effect of ELF-EMF on mitochondrial protein expression

Variations in mitochondrial activity could lead to an altered pattern of protein expression in the organelle. We evaluated whether the electromagnetic field might modulate the total intracellular levels of selected mitochondria-targeting proteins by Western blot analysis of whole cellular lysates. Moreover the mitochondrial localization of these proteins was evaluated after subcellular fractionation and mitochondrial purification. Nuclear extracts were also analysed in order to assess whether the possible effects of ELF-EMF were merely mitochondrial or were exerted also on nuclear compartment. The results of Western blot analysis are shown in Figure 4; both after 3 days of treatment (Figure 4A) and 7 days (Figure 4B) of exposure to ELF-EMF, the SKBR3 cells responded decreasing mitochondrial localization of phospho-ERK, p53, and cytochrome c, whereas the nuclear distribution of these proteins resulted unchanged. Quantification of Western blotting analysis is shown in Figure 4C (3 days of exposure) and Figure 4D (7 days). It is of note that total expression was not modulated. The effects of ELF-EMF on the analyzed proteins therefore seemed to be exerted specifically on their mitochondrial pools. Total ERK expression was not modulated in any fraction, demonstrating that the electromagnetic fields affect only the active phosphorylated form of ERK. The expression of the oncogenic protein receptor ErbB2/c-Neu is amplified in SKBR3 and it is a strong modulator of cell growth. ErbB2/c-Neu levels did not change in our assay, although under the influence of electromagnetic fields the SKBR3 cells were refrained in their growth, and the unaltered expression ruled out that the effects of ELF-EMF on proliferation could be mediated by receptor downregulation.

Figure 4. Evaluation of the effects of electromagnetic fields on protein expression. SKBR3 cells were exposed for 3 days (A, C) or 7 days (B, D) to the ELF-EMF and then the modulation of protein expression was analyzed in a panel of proteins by Western blotting of total lysates (TOT), mitochondrial extracts (MIT) and nuclear fractions (N). VDAC levels in total and mitochondrial fractions and PARP expression in nuclear extracts were used as internal controls for protein loading. (A, B) The blots are representative of a set of three independent experiments. (C, D) Bands were quantified, normalized for loading as a ratio to VDAC or PARP expression and data plotted on graph as values relative to control. Data represent the mean ± SD of three independent experiments. *$p < 0.05$ compared to control.



Discussion

The effects of ELF-EMF on cell growth are controversial, both in vitro and in vivo. A positive or negative modulation has been described depending on cellular model, field characteristics, and exposure times. Even when the studies on cancer cells have reported that ELF-EMF inhibits tumor growth, the molecular mechanisms sustaining the biological effects have been investigated with a scattered and cell-specific approach. Our goal was to characterize in vitro cell growth and modulation of cell metabolism when different models of cancer were continuously exposed to ELF-EMF of very low intensity. Long-term effects of ELF-EMF were thus evaluated, both on proliferation and mitochondrial activity. Differently from few previous studies, that tested mitochondrial pathways involved in apoptosis or ROS generation, we have considered the impact of mitochondrial activity on cancer cell metabolism and proliferation, an aspect of mitochondrial physiology that is emerging from latest studies (Park et al. 2010 Park J, Kusminski CM, Chua SC, Scherer PE. 2010. Leptin receptor signaling supports cancer cell metabolism through suppression of mitochondrial respiration in vivo. Am J Pathol 177:3133–3144.[CrossRef], [PubMed], [Web of Science ®], Santidrian et al. 2013 Santidrian AF, Matsuno-Yagi A, Ritland M, Seo BB, LeBoeuf SE, Gay LJ, Yagi T, Felding-Habermann B. 2013. Mitochondrial complex I activity and NAD+/NADH balance regulate breast cancer progression. J Clin Invest 123:1068–1081., Consiglio et al. 2014 Consiglio M, Destefanis M, Morena D, Foglizzo V, Forneris M, Pescarmona G, Silvagno F. 2014. The vitamin D receptor inhibits the respiratory chain, contributing to the metabolic switch that is essential for cancer cell proliferation. PLoS One 9:e115816.). The cancer cells employed in this study responded to electromagnetic exposure decreasing their proliferation and increasing their respiratory activity. The enhancement of mitochondrial membrane potential was reversible and was not accompanied by the increase in transcription of electron transport chain proteins. We concluded that the effects triggered by electromagnetic field were not exerted on transcription and translation of respiratory complexes, but on their activity. Since the pioneering work of Mitchell that led to the chemiosmotic theory (Mitchell 1961 Mitchell P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature 191:144–148.), it is well known that respiratory chain activity and oxidative phosphorylation are coupled, and that the increased energy demand in terms of ATP drives the increase in respiratory rate. The exposure of the cells to ELF-EMF did not change ATP levels, thus we could rule out that the observed increase of mitochondrial membrane potential was related to an inhibitory effect on ATP synthase; rather, the results of this study suggest that the ELF-EMF can either reduce proton leaks or can influence some ATP-demanding events, triggering a

compensatory increase of mitochondrial respiratory activity. The nature of these events has not been investigated in this work, and remains to be elucidated in future studies. However, taking into account the effects of electromagnetic field on calcium homeostasis described in literature, it is possible that the modulation of calcium influx could be responsible for an increased energy expenditure provoked by ELF-EMF.

In the present work we have demonstrated that ELF-EMF exposure enhances respiratory activity and reduces growth rate of cancer cells. Because our recent work (Consiglio et al. 2014 Consiglio M, Destefanis M, Morena D, Foglizzo V, Forneris M, Pescarmona G, Silvagno F. 2014. The vitamin D receptor inhibits the respiratory chain, contributing to the metabolic switch that is essential for cancer cell proliferation. PLoS One 9:e115816.) and few other reports (Park et al. 2010 Park J, Kusminski CM, Chua SC, Scherer PE. 2010. Leptin receptor signaling supports cancer cell metabolism through suppression of mitochondrial respiration in vivo. Am J Pathol 177:3133–3144., Santidrian et al. 2013 Santidrian AF, Matsuno-Yagi A, Ritland M, Seo BB, LeBoeuf SE, Gay LJ, Yagi T, Felding-Habermann B. 2013. Mitochondrial complex I activity and NAD+/NADH balance regulate breast cancer progression. J Clin Invest 123:1068–1081.) support the idea that increasing respiratory chain activity hinders tumor growth in cancer cells, we found a metabolic link that explains the negative effects of electromagnetic fields on cancer cellular proliferation: It is reasonably possible that the intensified activity of the respiratory chain stimulates the TCA cycle and the oxidative catabolism, with a net disadvantage for biosynthetic pathways, and the cancer cells are not supported in their demand of mitochondrial biosynthetic intermediates essential to proliferation.

The modulation of mitochondrial activity can influence protein pools resident in mitochondria, acting either on their import, activity or degradation. Protein import in mitochondria can be influenced by the metabolic activity of the organelle. For example the protein import machinery is intimately connected to the energetic state of mitochondria (Harbauer et al. 2014 Harbauer AB, Zahedi RP, Sickmann A, Pfanner N, Meisinger C. 2014. The protein import machinery of mitochondria-a regulatory hub in metabolism, stress, and disease. Cell Metab 19:357–372.). Also mitochondrial proteolytic activity resents the local conditions. For example the expression of PINK1 (PTEN-induced putative kinase 1) in individual mitochondria is regulated by voltage-dependent proteolysis (Narendra et al. 2010 Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, Cookson MR, Youle RJ. 2010. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biology 8:e1000298.). Therefore we sought to investigate whether mitochondrial modulation triggered by ELF-EMF exposure could impact on mitochondrial expression of some proteins. We analyzed a set of proteins which are either notoriously affected by electromagnetic fields in their signaling pathways: ERK1/2 (Martinez et al. 2012 Martinez MA, Ubeda A, Cid MA, Trillo MA. 2012. The proliferative response of NB69 human neuroblastoma cells to a 50 Hz magnetic field is mediated by ERK1/2 signaling. Cell Physiol Biochem 29:675–686.), or proteins which are involved in cell cycle and proliferation control: HER-2/Neu and p53. The amplification of the proto-oncogene HER-2/Neu and the overexpression of the encoded transmembrane protein p185 erbB2/c-Neu potently drives proliferation in SKBR3 breast cancer cells, whereas p53 plays a role in cell cycle arrest and apoptosis, and some effects of ELF-EMF on p53 expression have been reported (Tian et al. 2002 Tian F, Nakahara T, Yoshida M, Honda N, Hirose H, Miyakoshi J. 2002. Exposure to power frequency magnetic fields suppresses X-ray-induced apoptosis transiently in Ku80-deficient xrs5 cells. Biochem Biophys Res Commun 292:355–361.). Moreover mitochondrial levels of cytochrome c were evaluated as a marker of mitochondrial permeability mediated by the permeability transition pore (PTP). By Western blotting analysis we found reduced levels of pERK, p53 and cytochrome c in mitochondrial fractions of treated cells, whereas total expression and nuclear levels of these proteins were unaffected by ELF-EMF exposure.

The extracellular signal-regulated kinase 1/2 (ERK1/2) cascade is a central signaling pathway that regulates a wide variety of stimulated cellular processes, including mainly proliferation, differentiation, apoptosis and stress response. Besides the well known cytoplasmic and nuclear targeting, ERK1/2 molecules migrate also into distinct subcellular compartments, where they conduct specific functions. The mitochondrial localization of ERK1/2 has been described (Zhu et al. 2003 Zhu JH, Guo F, Shelburne J, Watkins S, Chu CT. 2003. Localization of phosphorylated ERK/MAP kinases to mitochondria and autophagosomes in Lewy body

diseases. Brain Pathol 13:473–481., Alonso et al. 2004 Alonso M, Melani M, Converso D, Jaitovich A, Paz C, Carreras MC, Medina JH, Poderoso JJ. 2004. Mitochondrial extracellular signal-regulated kinases 1/2 (ERK1/2) are modulated during brain development. J Neurochem 89:248–256.) and its role in mitochondria seems to be complex and controversial (Wortzel and Seger 2011 Wortzel I, Seger R. 2011. The ERK cascade: Distinct functions within various subcellular organelles. Genes Cancer 2:195–209.). It is known that redox condition modifies the activation of ERK1/2 (Kuruganti et al. 2002 Kuruganti PA, Wurster RD, Lucchesi PA. 2002. Mitogen activated protein kinase activation and oxidant signaling in astrocytoma cells. J Neurooncol 56:109–117., Samavati et al. 2002 Samavati L, Monick MM, Sanlioglu S, Buettner GR, Oberley LW, Hunninghake GW. 2002. Mitochondrial K (ATP) channel openers activate the ERK kinase by an oxidant-dependent mechanism. Am J Physiol Cell Physiol 283:C273–281.). In brain the effects of exogenous or endogenously formed H_2O_2 on mitochondrial ERK were explored (Alonso et al. 2004 Alonso M, Melani M, Converso D, Jaitovich A, Paz C, Carreras MC, Medina JH, Poderoso JJ. 2004. Mitochondrial extracellular signal-regulated kinases 1/2 (ERK1/2) are modulated during brain development. J Neurochem 89:248–256.); the results of this study showed that H_2O_2 decreases mitochondrial phospho-ERK1/2 levels, reversed by catalase addition. Effects of H_2O_2 may be exerted on cysteine residues of the mitochondrial upstream kinase MEK1/2 (Kim et al. 2001 Kim KY, Rhim T, Choi I, Kim SS. 2001. N-acetylcysteine induces cell cycle arrest in hepatic stellate cells through its reducing activity. J Biol Chem 276:40591–40598.). High H_2O_2 may also contribute to regulate the mitochondrial ERK availability; stress-activated ERK undergo to ubiquitination and degradation (Lu et al. 2002 Lu Z, Xu S, Joazeiro C, Cobb MH, Hunter T. 2002. The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. Mol Cell 9:945–956.), a process that may take place in mitochondria (Cogswell et al. 2003 Cogswell PC, Kashatus DF, Keifer JA, Guttridge DC, Reuther JY, Bristow C, Roy S, Nicholson DW, Baldwin AS. 2003. NF-kappa B and I kappa B alpha are found in the mitochondria. Evidence for regulation of mitochondrial gene expression by NF-kappa B. J Biol Chem 278:2963–2968.). In our experimental set cancer cells exposed to ELF-EMF increase mitochondrial respiratory activity; because mitochondria are the main cell producers of H_2O_2 at complexes I and III (Boveris and Cadenas 1997 Boveris A, Cadenas E. 1997. Cellular sources and steady-state levels of reactive oxygen species. In: Biadasz Clerch L, Massaro DJ, editors. Oxygen, gene expression and cellular function. New York: Marcel Dekker Inc. pp. 1–25.), the enhanced ROS production would explain the reduced levels of phospho-ERK observed in mitochondrial fractions.

Recent works have described the mitochondrial localization of p53. In this organelle p53 associates with VDAC (Ferecatu et al. 2009 Ferecatu I, Bergeaud M, Rodríguez-Enfedaque A, Le Floch N, Oliver L, Rincheval V, Renaud F, Vallette FM, Mignotte B, Vayssière JL. 2009. Mitochondrial localization of the low level p53 protein in proliferative cells. Biochem Biophys Res Commun 387:772–777.), one of the members of the permeability transition pore. Moreover it has been demonstrated that mitochondrial permeability transition affects p53 translocation (Liu et al. 2008 Liu J, St Clair DK, Gu X, Zhao Y. 2008. Blocking mitochondrial permeability transition prevents p53 mitochondrial translocation during skin tumor promotion. FEBS Lett 582:1319–1324.). p53 plays a role in mtDNA transcription and translation by either binding directly (Heyne et al. 2004 Heyne K, Mannebach S, Wuertz E, Knaup KX, Mahyar-Roemer M, Roemer K. 2004. Identification of a putative p53 binding sequence within the human mitochondrial genome. FEBS Lett 578:198–202., Kulawiec et al. 2009 Kulawiec M, Ayyasamy V, Singh KK. 2009. p53 regulates mtDNA copy number and mitocheckpoint pathway. J Carcinog 8:8.) or indirectly via mitochondrial transcription factor A (Tfam) to mtDNA (Yoshida et al. 2003 Yoshida Y, Izumi H, Torigoe T, Ishiguchi H, Itoh H, Kang D, Kohno K. 2003. p53 physically interacts with mitochondrial transcription factor A and differentially regulates binding to damaged DNA. Cancer Res 63:3729–3734.). Acute exercise induces the translocation of p53 to mitochondria, where p53 positively affect mtDNA transcription (Saleem and Hood 2013 Saleem A, Hood DA. 2013. Acute exercise induces tumour suppressor protein p53 translocation to the mitochondria and promotes a p53-Tfam-mitochondrial DNA complex in skeletal muscle. J Physiol 591:3625–3636.). The boosted respiratory activity might exert a negative feedback on mitochondrial p53 expression, a regulatory mechanism which is typical of tightly controlled events. This could explain a reduction in p53 levels observed in our experimental conditions. On the other hand, in response to ELF-EMF the increase in respiratory activity and ROS production could modify the opening frequency of PTP, which exhibits the features of a voltage- and pH-gated channel, modulated by the redox and phosphate

potentials (Ichas and Mazat 1998 Ichas F, Mazat JP. 1998. From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. Biochim Biophys Acta 1366:33–50.). It is possible that a modulation in PTP opening triggers a different import/export rate of p53. In fact it is well known that PTP opening is involved in the release of proapoptotic factors such as cytochrome c from mitochondria; actually also in our experimental setting we display a reduced presence of cytochrome c in mitochondrial fraction, supporting therefore the hypothesis that electromagnetic fields could be modulating the opening of PTP. However, this stimulus does not trigger apoptosis, because the increase of DNA fragmentation or the sub-G0 peak was not detected by flow cytometry analysis of cells exposed to electromagnetic field (data not shown). Moreover apoptotic cell death is preceded by mitochondrial malfunction including collapse of mitochondrial membrane potential and in our treated cell we show the increase in membrane potential.

The reduced levels of mitochondrial phospho-ERK and p53 could be simply the hallmarks of the increased mitochondrial activity and permeability, likewise the loss of mitochondrial cytochrome c, or their altered expression could play a role in the inhibition of proliferation, with mechanisms not investigated yet.

In conclusion, this study showed for the first time that the effects exerted by ELF-EMF on cancer cells are mediated by the modulation of mitochondrial activity. We reported that the inhibited proliferation of several cancer cell lines was accompanied by the increased mitochondrial respiration, reversibly modulated possibly by energy demand, and by a downregulation of mitochondrial protein expression. Although further and more comprehensive studies are still required to elucidate the molecular mechanisms responsible for mitochondrial activation in ELF-EMF, this work demonstrates a beneficial effect of exposing cancer cells to electromagnetic fields, in agreement with other studies (reviewed in Verginadis et al. 2012 Verginadis I, Velalopoulou A, Karagounis I, Simos Y, Peschos D, Karkabounas S, Evangelou A. 2012. Beneficial effects of electromagnetic radiation in cancer. In: Bashir SO, editor. Electromagnetic radiation. InTech, DOI: 10.5772/35456. ISBN: 978-953-51-0639-5. Available from: http://www.intechopen.com/books/electromagnetic-radiation/beneficial-effects-of-electromagnetic-radiation-in-cancer). Because it has been reported the lack of adverse effects of ELF-EMF on proliferation of non–cancerous cells (Tofani et al. 2001 Tofani S, Barone D, Cintorino M, De Santi MM, Ferrara A, Orlassino R, Ossola P, Peroglio F, Rolfo K, Ronchetto F. 2001. Static and ELF magnetic fields induce tumor growth inhibition and apoptosis. Bioelectromagnetics 22:419–428., Huang et al. 2014b Huang CY, Chuang CY, Shu WY, Chang CW, Chen CR, Fan TC, Hsu IC. 2014b. Distinct epidermal keratinocytes respond to extremely low-frequency electromagnetic fields differently. PLoS One 9:e113424.) this work opens a new investigation field valuable for future clinical applications.